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(54) Title: P43 ANTI-TUMOR THERAPEUTIC AGENT AND THREE DIMENSIONAL STRUCTURE OF ITS CYTOKINE DOMAIN

(57) Abstract: The object of the present invention is to provide an anti-tumor and anti-angiogenic agent of p43 (SEQ ID NO : 2) consisting of two domains, N-terminal domain (146 amino acids) and C-terminal domain (166 amino acids), wherein C-terminal domain consists of 11 β-strands forming a structural core and 3 flanking α-helices.

p43 ANTI-TUMOR THERAPEUTIC AGENT AND THREE DIMENSIONAL STRUCTURE OF ITS CYTOKINE DOMAIN

TECHNICAL FIELD

The present invention relates to the anti-tumor and anti-angiogenic activities of p43 and the three dimensional structure of its cytokine domain.

BACKGROUND ART

Endothelial monocyte activating polypeptide II (EMAP II) was originally isolated from supernatant of the cultured mathylcholathrene A induced fibrosarcoma cells (1). The recent report showed that EMAP II suppressed primary and metastatic tumor growth and triggered apoptosis in proliferating endothelial cells without apparent adverse effect on normal tissues (2). Thus, EMAP II is expected to have a therapeutic potential as an anti-angiogenic factor.

This protein is released from its precursor, p43, upon apoptosis and triggers pro-inflammatory response (3). Its precursor, p43 (pro-EMAP II), consists of 314 aa and is normally associated with the multi-tRNA synthetase complex to enhance the catalytic activity of the bound enzyme (4). However, it is cleaved by the activated caspase-7 at ASTD147/S in apoptotic condition to separate its C-terminal domain,

EMAP II (5).

Interestingly, the sequence of EMAP II shows homology to the domains present in several different aminoacyl-tRNA synthetases (6) and could bind tRNAs while it is not homologous to any known cytokines. The N-terminal synthetic 15 aa peptide was shown to be responsible for the cytokine activities of EMAP II (7). Thus, this N-terminal peptide may determine the interaction with its cellular receptors yet to be identified.

While the EMAP II domain has been extensively studied for its cytokine activities, the structure and function of the N-terminal domain of p43 has not been understood. We have previously shown that this domain is responsible for the interaction with the N-terminal non-catalytic extension of human arginyl-tRNA synthetase (4). We also showed that both of the domains in p43 are required to give a stimulatory effect on the interacting arginyl-tRNA synthetase.

The structure and maturation of EMAP II is reminiscent of 14.5 kDa IL-1b that exerts pro-inflammatory response. This cytokine is generated by the cleavage of ICE (caspase-1) from its 33 kDa precursor (pre-IL-1b) that is inactive. Although the maturation process of the two cytokines are similar, the precursor of EMAP II, p43, appears to be different from pre-IL-1b in that it is associated with the multi-tRNA synthetase.

Although the sequence and biological activities of EMAP II were extensively studied previously, the functional meaning of its precursor, p43 (pro-EMAP II), was not well understood. However, there are some data in the previous reports implicating that p43 itself may work as a

cytokine.

First, the proteins from the secreted meth A cells were separated by chromatography and they were fractionated based on the ability to induce tissue factor activity in endothelial cells. Among the active fractions, the fraction containing EMAP II (22 kDa) showed additional active peak at the higher molecular weight (about 40 kDa). Although this protein was assumed to be EMAP I or VPF/VEGF (1), it is possible that this peak may contain p43.

Second, the full length p43 was released from meth A cells throughout the cultivation (3) although the functional reason for this release was not explained. Based on these results, we thought that the full-length p43 could be an active cytokine.

U. S. Pat. No. 5,641,867 disclosed the identification and biological activities of EMAP II. Although we are aware that EMAP II works as an active pro-inflammatory cytokine, we compared p43 with EMAP II for various cytokine activities in the present invention and found that p43 itself exerts more potent cytokine activity than its proteolytic product, EMAP II.

In addition, we also solved the three dimensional structure of EMAP II at 1.8 Å to understand the molecular mechanism of its biological activities. These findings would significantly change and improve our understanding of the process and functional significances of this novel cytokine and provide important information on the clinical application of this protein as an anti-tumor or anti-angiogenic agent.

DISCLOSURE OF INVENTION

The object of the present invention is to provide an anti-tumor and anti-angiogenic agent of p43 (SEQ ID NO : 2) consisting of two domains, N-terminal domain (146 amino acids) and C-terminal domain (166 amino acids), wherein C-terminal domain consists of 11 β-strands forming a structural core and 3 flanking α-helices.

The other object of the present invention is to provide 11 β-strands essentially consists of β1 (residues 10-21), β2 (residues 28 to 34), β3 (residues 40 to 46), β4 (residues 59 to 66), β5i (residues 70 to 72), β6i (residues 75 to 77), β7 (residues 79 to 85), β8 (residues 90 to 92), β9 (residues 103 to 106), β10 (residues 132 to 134) and β11 (residues 140 to 142), and 3 α-helices essentially consists of α1 (residues 53 to 56), α2 (residues 119 to 123) and α3 (residues 124 to 130) when cell dimensions are a = 134.01 Å, b = 38.34 Å, c = 80.99 Å, a = g = 90° and b = 112.90° and crystals have a non-crystallographic two-fold symmetry at omega = 36.7° , phi = 286.4° and kappa = 180° .

The further object of the present invention is to provide a process for preparing p43 by the expression of cDNA clone (SEQ ID NO : 1) comprising the steps of :

i) amplifying the cDNA clone by using two sets of primers (SEQ ID NO : 3 and 4) and (SEQ ID NO : 5 and 6) ;

ii) assembling the expression vector by inserting amplified cDNA clone ;

iii) expressing the cDNA in the host cell ; and

iv) selecting and purifying the obtained protein p43.

The present invention also provides the pharmaceutical preparation having the amount of p43 prepared by the steps disclosed as above.

The present invention also includes the use of p43 to isolate its cellular receptor as well as the use of three dimensional structure of EMAP II to design any peptide and small chemical derivatives for pharmaceutical purpose.

BRIEF DESCRIPTION OF DRAWINGS

FIG 1 shows the cDNA and amino acid sequences of human p43.

FIG 2 shows the SDS-gel electrophoresis bands of full length, N-terminal domain and C-terminal domain of p43.

FIG 3 shows the effect of p43-F, p43-N and p43-C on murine tumor regression compared to control.

FIG 4 shows the effect of p43 and EMAP II on the production of TNF-a, IL-6 and IL-8.

FIG 5 shows the effect of p43 and EMAP II on the secretion of matrix matalloprotease-9 (MMP-9).

FIG 6 shows the *in vitro* motility assay of human melanoma cells.

FIG 7 shows the overall structure of human EMAP II.

FIG 8 shows the structural homology of EMAP II compared to other cytokines.

BEST MODE FOR CARRYING OUT THE INVENTION

A protein of apparent molecular weight of 43 kDa (p43) is associated with the macromolecular tRNA synthetase complex to enhance the catalytic activity of the bound enzyme. This protein is a precursor of endothelial monocyte activating polypeptide II (EMAP II) that is generated by proteolysis with caspase-7 upon apoptosis.

In the present invention, we found that p43 and its C-terminal cytokine domain (EMAP II) induced regression of fibrosarcoma in the immunocompromised mouse while its N-terminal domain did not.

Further, *in vitro* analyses showed that p43 is better potent cytokine as determined by the induction of TNF-a, IL-6, IL-8 and matrix metalloprotease-9 or by its activity of chemotaxis.

We also determined the three dimensional structure of the C-terminal cytokine domain of p43 at 1.8 Å. The protein consists of two distinct structural domains. The structure consists of two distinct sub-domains and the N-terminal region forms an oligonucleotide binding fold (OB fold). A part of this N-terminal region also showed a limited structural homology to a few other cytokines such as RANTES, human monocyte chemoattractant protein, and neutrophil-activating peptide-2.

Thus, in the present invention, we disclose that p43 itself may

function as a novel cytokine with pro-apoptotic activity and can be employed as an anti-tumorigenic agent. We also solved the three dimensional structure of the C-terminal domain responsible for the cytokine activity.

FIG 2 shows the SDS-gel electrophoresis bands of full length, N-terminal domain and C-terminal domain of p43.

Purified recombinant proteins of the full-length p43 (312 amino acids) and its N-terminal 146 amino acids and C-terminal 166 amino acids polypeptides.

The structure of human pro-EMAPII was divided into the N- and C-terminal domains at D146 that is the cleavage site by caspase-7. The cDNA encoding the full-length p43 was isolated from pM338 using Nde1 and Xho1. cDNAs for the N- and C-terminal domains of pro-EMAPII were isolated by PCR using the primer pairs of R1EF (SEQ ID NO : 3) / S1ENB (SEQ ID NO : 4) and R1ECF (SEQ ID NO : 5) / S1EB (SEQ ID NO : 6) respectively.

The obtained cDNAs were cloned into pET28a using Nde1/Xho1 and EcoR1/Sal1, respectively. The cells expressing the recombinant proteins were harvested and resuspended in 20 mM KH_2PO_4, 500 mM NaCl (pH 7.8), 2 mM 2-mercaptoethanol and lysed by ultrasonication. After centrifugation of the lysate at 25,000 g, the supernatants were obtained and subjected to nickel affinity chromatography following the manufacturer's instruction (Invitrogen).

The purity of the purified recombinant proteins was determined by SDS-gel electrophoresis.

FIG 3 shows the effect of p43-F, p43-N and p43-C on murine tumor regression compared to control.

Specific pathogen free C3H/He mouse was used to determine the effect of p43 and its separate domains on tumor growth as described previously (8). Methylcholanthrene A induced fibrosarcoma cells (2 x 105 cells) were intradermally injected into the dorsal skin of mouse.

When tumors reached about 100 mm^3 (day 0), they were directly injected one time with 0.1 ml of vehicle solution (PBS with 0.1 % BSA) alone or containing each of the full-length, N- and C-terminal domains of p43 (100 ug/ml). Tumor volumes were defined by (length x width x height)/2 and measured 2 and 4 days after injection. The effect of p43 on tumor growth was determined by the percent of the initial volume and the data were subjected to t-Test.

FIG 4 shows the effect of p43 and EMAP II on the production of TNF-a, IL-6 and IL-8.

The induction of cytokines was determined by Enzyme-Linked Immunosorbent Assay (ELISA). TNF-a, IL-6 and IL-8 were measured by the sandwich ELISA systems (Endogen Inc., USA). The detection limits were < 3 pg/ml for IL-6, < 2 pg/ml for IL-8, and < 5 pg/ml for TNF-a.

FIG 5 shows the effect of p43 and EMAP II on the secretion of matrix matalloprotease-9 (MMP-9).

The activity of MMP-9 (matrix matalloprotease-9) was determined by gelatin zymography as described previously (9). Human THP-1

monocytic cells were cultured in the conditioned media and treated with each of the p43 proteins at the indicated concentrations. The MMP-9 secreted from the cells was determined by performing substrate gel electrophoresis with modifications.

Samples in SDS-PAGE loading buffer (without reducing agent) were applied to 10% Tris-glycine polyacrylamide zymogram gels containing 0.1% gelatin and separated by electrophoresis. The gels were subsequently washed with renaturing buffer for 30 min followed by developing buffer (50 mmol/L Tris-HCl, pH 7.5, 0.15 mol/L NaCl, 10 mmol/L $CaCl_2$, and 0.02% NaN_3) for 30 min at room temperature.

The zymogram gels were subsequently incubated overnight at 37℃ in fresh developing buffer. The gel was then stained with Coomassie brilliant blue R-250 solution.

FIG 6 shows the *in vitro* motility assay of human melanoma cells.

Chemotactic activity of p43 and EMAP II was determined in 48 well microchemotaxis chambers with gelatin-coated 8 μm polyvinylpyrrolidine-free polycarbonate filters (Neuroprobe, Cabin John, USA) as described previously (10). The experiments were performed in triplicate.

FIG 7 shows the overall structure of human EMAP II.

EMAP II was stored at 10 mg/ml in 20 mM Tris-HCl, pH 8.0, 0.5 mM 2-mercaptoethanol, 1 mM EDTA and 100 mM KCl. The crystals of EMAP II were grown over a period of 3 days at 21℃ in hanging drops formed by mixing 3 ml of the protein solution and 3 ml of a reservoir

solution, which consisted of 100 mM sodium acetate, pH 4.6, 20% (w/v) PEG 4000 and 15 mM $MgCl_2$.

Cell dimensions are a = 134.01 Å, b = 38.34 Å, c = 80.99 Å, a = g = 90° and b = 112.90° . It contains 2 molecules per asymmetric unit and 64% solvent.

These crystals have a non-crystallographic two-fold symmetry at omega = 36.7° , phi = 286.4° and kappa = 180° , described in POLRFN [CCP4, 1994 #7718].

Native and heavy atom derivative data were collected at room temperature on a RAXIS IV detector with a Rigaku RU200 rotating anode (Cu-K) X-ray generator operated at 50 kV and 100 mA. All data were processed with DENZO and SCALEPACK (11).

FIG 8 shows the structural homology of EMAP II compared to other cytokines.

The structural homology search program DALI (12) identified that the N-terminal 45 residues of EMAP II (residues 13 to 57) have limited structural homologies with monomers of chemokines, such as RANTES (residues 22 to 61) (13), human monocyte chemoattractant protein (residues 25 to 65) (14), and neutrophil-activating peptide-2 (residues 38 to 79) (15).

In EMAP II, strands b-b3 and helix a1 form a homologous structural domain of monomers of these chemokines even though the primary sequence of EMAP II shows no homology. The Cas of those chemokine structural motifs in RANTES, human monocyte

chemoattractant protein and neutrophil-activating peptide-2 could be superimposed onto the corresponding region of EMAP II with r.m.s.d's of 1.78 Å for 30 atoms, 1.75 Å for 23 atoms and 2.5 Å for 21 atoms, respectively.

The present invention will be more specifically explained by the following example. However, it should be understood that the example is intended to illustrate but not in any manner to limit the scope of the present invention.

EXAMPLES

(Example 1) Tumor regression effect of p43

The full-length p43 consists of 312 aa (FIG 1) and is associated with the multi-tRNA synthetase complex. It is cleaved at D147 by caspase-7 to release 22 kDa EMAPII. Although EMAP II was extensively studied for its cytokine activity, the activity of its precursor, p43, have not been investigated. Here, we thus compared p43 and EMAP II for the various cytokine activities. The full-length of p43 and its N- and C-terminal domains were expressed as His tag proteins and purified using nickel affinity purification. These recombinant proteins were purified to near homogeneity as determined by gel electrophoresis (FIG 2). To test whether these purified proteins affect the tumor growth, we first transplanted methalcholanthrene A-induced fibrosarcoma into the immunocompromised mouse. Then, each of the purified proteins was intratumorally injected in triplicate and the volume of the

treated tumors was determined at timer interval. To our surprise, the sizes of the tumors were decreased by the treatment of p43 or EMAP II while tumor growth was not affected by the treatment of the N-terminal domain of p43 or vehicle alone (FIG 3). These results suggest that p43 but not its N-terminal domain, could be an active cytokine as its C-terminal EMAP II domain.

(Example 2) Induction of cytokines by p43

The cytokine activity of p43 was further investigated by various in vitro systems. Human monocytic THP-1 cells were cultured and treated either with the indicated amounts of p43 or EMAP II. The production of IL-6, -8 and TNF-a from the treated cells was determined by ELISA using their respective antibodies. The induction levels of these cytokines by the treatment of p43 at 50 nM were similar to those with EMAP II at 500 nM, indicating that p43 is more potent in the induction of these cytokines (FIG 4). Then, the induction of the secretion of matrix metalloprotease-9 with p43 or EMAP II was determined by gelatin zymography. The supernatant of the human THP-1 was obtained after the treatment of p43 or EMAP II at the indicated concentrations. The cells treated with either one of the proteins secreted MMP-9 while the control untreated cells did not (FIG 5). Again, the MMP-9 secretion was more strongly induced by the treatment of p43 compared to EMAP II. We then compared the chemotactic activity of the two proteins using in vitro motility assay of human malanoma cells. p43 induced chemotactic response of melanoma cells in a dose response manner from 1 to 10 ng/ml while EMAP II did not (FIG 6). All of these results indicate that p43 is a more potent cytokine than its proteolytic product,

EMAP II.

(Example 3) Three dimensional structure of EMAP II domain

To gain an insight into the structural reason for the cytokine activity of this region, we have purified the C-terminal domain of p43 (EMAP II) and its three dimensional structure was determined by X-ray crystallography. The crystal structure of EMAP II was solved using the single isomorphous replacement method. The final model contains two molecules per asymmetric unit (residues 3-166) and 193 water molecules, refined at 1.8 A resolution. The EMAP II structure consists of 11 β -strands forming a structural core and 3 flanking α-helices (FIG 7) : the strands β1 (residues 10-21), β2 (residues 28 to 34), β3 (residues 40 to 46), β4 (residues 59 to 66), β5i (residues 70 to 72), β6i (residues 75 to 77), β7 (residues 79 to 85), β8 (residues 90 to 92), β9 (residues 103 to 106), β10 (residues 132 to 134), β11 (residues 140 to 142), and the α -helices α1 (residues 53 to 56), α2 (residues 119 to 123), α3 (residues 124 to 130). The structure can be divided into the N- and C-terminal regions. The N-terminal region, consisting of the strands β1-7 and the helix α1, forms a distinct structural motif called the oligonucleotide/oligosaccharide binding-fold (OB fold) [Murzin, 1993 #7726], which is known to bind oligonuclotide and oligosaccharide. The OB fold of EMAP II has a five stranded Greek-key β-barrel (strands β 1-3, β4, and β7) that is capped by the short helix α1, which is located between the strands β3 and β4. The C-terminal region contains the strands β8-11, the helices α2-3, and several long loops. This C-terminal region contains longer loops compared to the N-terminal region and does not share a homology to any known structure.

(DISCUSSION)

Since EMAP II was first isolated from the cultured supernatant of the murine meth A fibrosarcoma cells, its biological activities have been extensively studied. This protein works as a novel mediator of proinflammatory response and chemoattractant for monocytes (1,7,8). Its therapeutic potential as an anti-tumor agent was also suggested by showing the suppressive effect on tumor growth and pro-apoptotic activity against the proliferating endothelial cells (2). Although EMAP II showed the strong proinflammatory cytokine activity, it did not appear to be an early mediator of inflammation because its production is only apparent at least 12 hrs after the onset of apoptosis (3).

EMAP II is generated by the cleavage of p43 at D147 by caspase-7 (5). Since EMAP II was an active cytokine, the biological activity of its precursor, p43, has not been well understood. We have previously shown that it is associated with the multi-tRNA synthetase complex and enhances the activity of the bound enzyme (4). In this work, we investigated whether p43 can also function as an active pro-inflammatory cytokine and found that it is a more potent cytokine than its cleavage product, EMAP II (FIG 3~7). Thus, the proteolytic cleavage of p43 is not to activate EMAP II but simply to release this domain. We also found that p43 is also released into extracellular matrix from the cultivated cells (data not shown).

To understand the molecular mechanism for the biological activity of EMAP II, we solved the three dimensional structure of EMAP II at 1.8

A (FIG 7). The determined structure of p43 showed that it contains a structural motif called OB fold that is responsible for oligonucleotide binding (16). The N-terminal region of EMAP II also showed the weak homology to the corresponding regions of other cytokines (FIG 8). This motif explains why it can bind to tRNA (6) and exert the cytokine activity.

Studies on murine and human EMAP II (7,17) suggest that the residues responsible for cytokine activity in human EMAP II are the residues from 6 to 18 (VSRLDLRIGCIIT). This peptide stimulated chemotaxis and increased cytosolic calcium in mononuclear phagocytes and polymorphonuclear leucocytes; whereas, the last 7 residues (residues 12 to 18, RIGCIIT) triggers only chemotaxis. The 13 residues for those cytokine activities lie mostly in a long stretch of strand b1. The Val6, Leu9, and Leu11 form a shallow hydrophobic pocket with neighboring residues, such as Phe107 and Leu132, in the solvent accessible surface. A short a-helix turn starts at the residue Val6. Residues 12 to 18, which are mediating chemotaxis, are positioned towards the end of strand $\beta 1$ that is flanked by strand $\beta 2$ and $\beta 4$. Interestingly, these last seven residues are separated from the first 6 residues by a loop consisting of residues 99 to 102. Three residues, Arg12 to Gly14, in the shorter sequence (RIGCIIT) are covered by the loop and only three residues (Cys15, Ile17, Thr18) expose their side chains to solvent. Peptides with substitutions on the position of Thr18 had no cytokine activities (17). This may imply that the residue Thr18 plays a crucial role in accessibility for receptor binding or other interactions for cytokine activity. Our finding that p43 is a more potent cytokine than EMAP II and the three dimensional structure of EMAP II would provide an important information on the clinical application of this novel cytokine.

(REFERENCES)

1. Kao, J., Ryan, J., Brett, J., Chen, J., Shen, H., Fan, Y.-G., Godman, G., Familletti, P., Wang, F., Pan, Y.-C., Stern, D., and Clauss, M. (1992) J. Biol. Chem. 267, 20239-20247

2. Schwarz, M. A., Kandel, J., Brett, J., Li, J., Hayward, J., Schwarz, R. E., Chappey, O., Wautier, J.-L., Chabot, J., Gerfo, P. L. and Stern, D. (1999) J. Exp. Med. 190, 341-353

3. Knies, U. E., Behrensdorf, H.A., Mitchell, C.A., Deutsch, U., Risau, W., Drexler, H.C.A., and Cluass, M. (1998) Proc. Natl. Acad. Sci. USA 95, 12322-12327

4. Park, S. G., Jung, K.H., Lee, J.S., Jo, Y.J., Motegi, H., Kim, S., and Shiba, K. (1999) J. Biol. Chem. 274, 16673-16676

5. Behrensdorf, H. A., van de Craen, M., Knies, U. E., Vandenabeele, P., and Clauss, M. (2000) FEBS Lett. 466, 143-147

6. Quevillon, S., Agou, F., Robinson, J. C., and Mirande, M. (1997) J. Biol. Chem. 272, 32573-32579

7. Kao, J., Fan, Y.-G., Haehnel, I., Brett, J., Greenberg, S., Clauss, M., Kayton, M., Houck, K., Kisiel, W., Seljelid, R., Burnier, J., and Stern, D. (1994) J. Biol. Chem. 269, 9774-7982

8. Kao, J., Houck, K., Fan, Y., Haehnel, I., Libutti, S. K., Kayton, M. L.,

Grikscheit, T., Chabot, J., Nowygrod, R., Greenberg, S., Kuang, W.-J., Leung, D. W., Hayward, J. R., Kisiel, W., Heath, M., Brett, J., and Stern, D. M. (1994) J. Biol. Chem. 269, 25106-25119

9. Birkedal-Hansen, H., and Taylor, R. E. (1982) Biochem. Biophys. Res. Commun. 107, 1173-1178

10. Aznavoorian, S., Stracke, M. L., Parson, J., McClanahan, J., and Liotta, L. A. (1996) J. Biol. Chem. 271, 3247-3254

11. Otwinowski, Z., and Minor, W. (1997) Methods Enzymol. 276, 307-326

12. Holm, L., and Sander, C. (1993) J. Mol. Biol. 233, 123-138

13. Wilken, J., Hoover, D., Thompson, D. A., Barlow, P. N., McSparron, H., Picard, L., Wlodawer, A., Lubkowski, J., and Kent, S. B. (1998) Chem. Biol. 6, 43-51

14. Lubkowski, J., Bujacz, G., Boque, L., Domaille, P. J., Handel, T. M., and Wlodawer, A. (1997) Nature Struct. Biol. 4, 64-69

15. Malkowski, M. G., Wu, J. Y., Lazar, J. B., Johnson, P. H., and Edwards, B. F. P. (1995) J. Biol. Chem. 270, 7077-7087

16. Murzin, A. G. (1993) EMBO J. 12, 861-867

17. Wakasugi, K., and Schimmel, P. (1999) J. Biol. Chem. 274, 23155-23159

WHAT IS CLAIMED IS :

1. An anti-tumor and anti-angiogenic agent of p43 (SEQ ID NO : 2) consisting of two domains, N-terminal domain (146 amino acids) and C-terminal domain (166 amino acids), wherein C-terminal domain consists of 11 β-strands forming a structural core and 3 flanking α-helices.

2. The anti-tumor and anti-angiogenic agent of p43 according to claim 1, wherein 11 β-strands essentially consists of β1 (residues 10-21), β2 (residues 28 to 34), β3 (residues 40 to 46), β4 (residues 59 to 66), β5i (residues 70 to 72), β6i (residues 75 to 77), β7 (residues 79 to 85), β8 (residues 90 to 92), β9 (residues 103 to 106), β10 (residues 132 to 134) and β11 (residues 140 to 142).

3. The anti-tumor and anti-angiogenic agent of p43 according to claim 1, wherein 3 α-helices essentially consists of α1 (residues 53 to 56), α2 (residues 119 to 123) and α3 (residues 124 to 130).

4. The anti-tumor and anti-angiogenic agent of p43 according to any one of claims 1~3, wherein cell dimensions are a = 134.01 Å, b = 38.34 Å, c = 80.99 Å, a = g = 90° and b = 112.90° and crystals have a non-crystallographic two-fold symmetry at omega = 36.7° , phi = 286.4° and kappa = 180° .

5. The process for preparing p43 by the expression of cDNA clone (SEQ ID NO : 1) comprising the steps of :
   i) amplifying the cDNA clone by using two sets of primers (SEQ ID NO : 3 and 4) and (SEQ ID NO : 5 and 6) ;
   ii) assembling the expression vector by inserting amplified cDNA

clone ;

iii) expressing the cDNA in the host cell ; and

iv) selecting and purifying the obtained protein p43.

6. The pharmaceutical preparation having the amount of p43 prepared by the steps disclosed in claim 5.

7. The use of p43 to isolate its cellular receptor.

8. The use of three dimensional structure of EMAP II to design any peptide and small chemical derivatives for pharmaceutical purpose.

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FIG 1

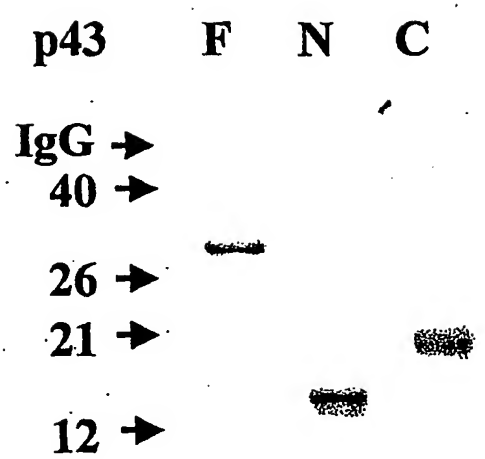

FIG 2

Effect of p43-F, -N and –C on Tumor Growth

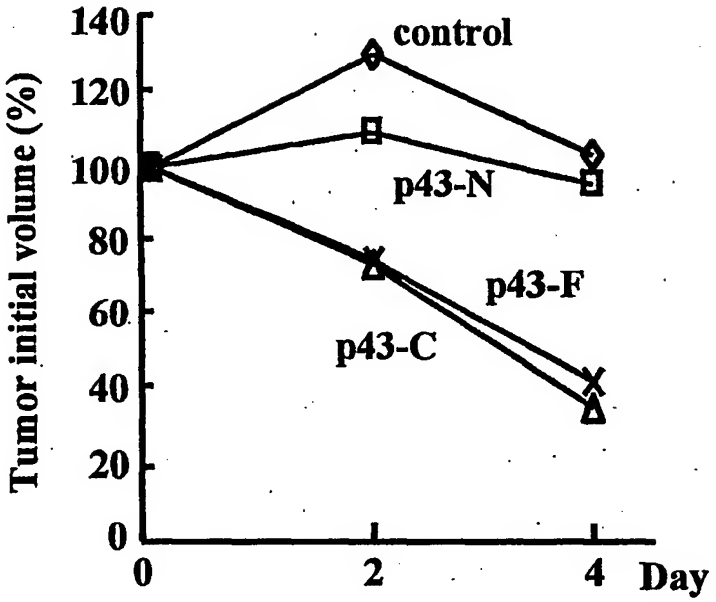

FIG 3

Cytokine Function of p43 and EMAP-II

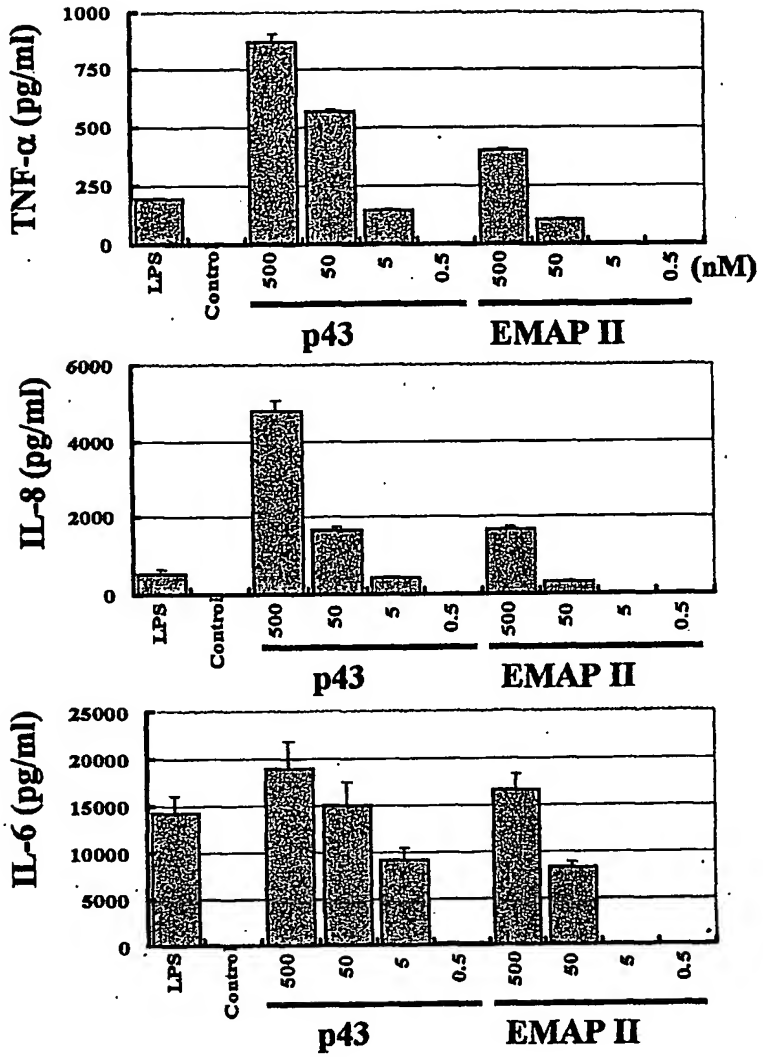

FIG 4

Induction of MMP-9 Secretion

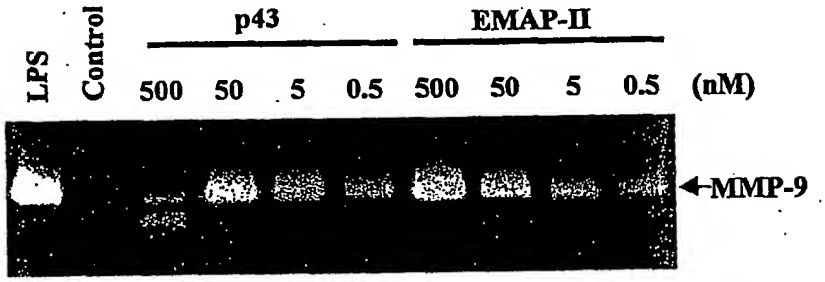

FIG 5

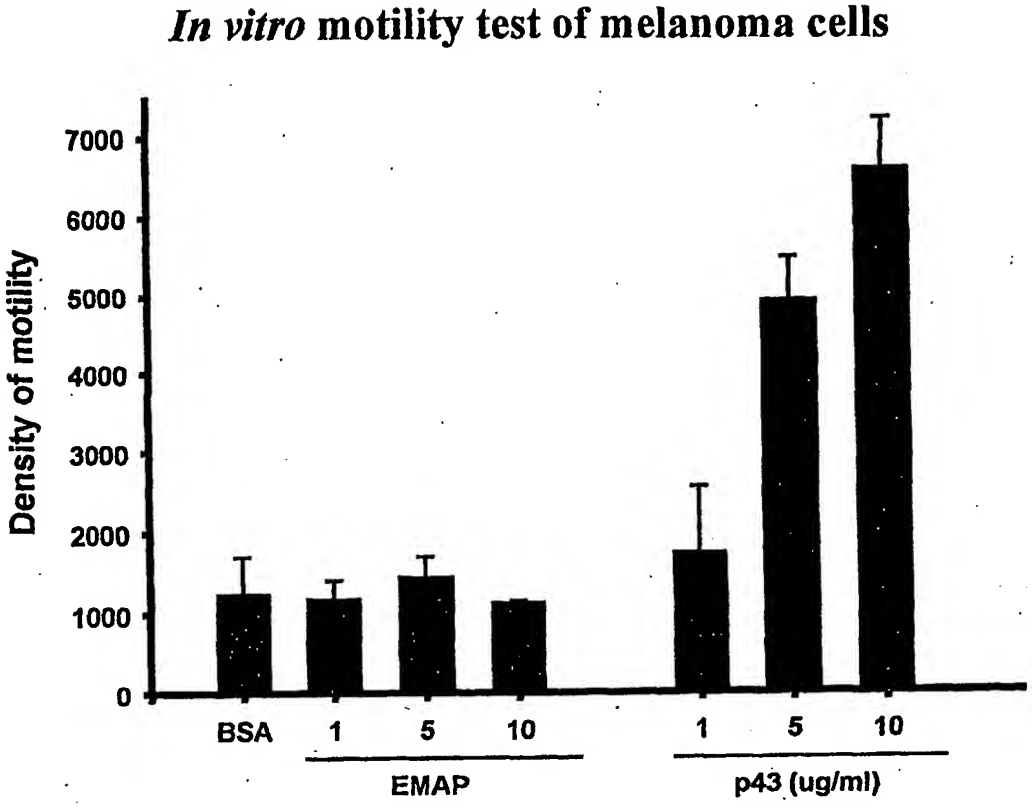
In vitro motility test of melanoma cells
Density of motility
7000
6000
5000
4000
3000
2000
1000
0
BSA
1
5
10
EMAP
1
5
10
p43 (ug/ml)

FIG 6

Overall structure of EMAP II

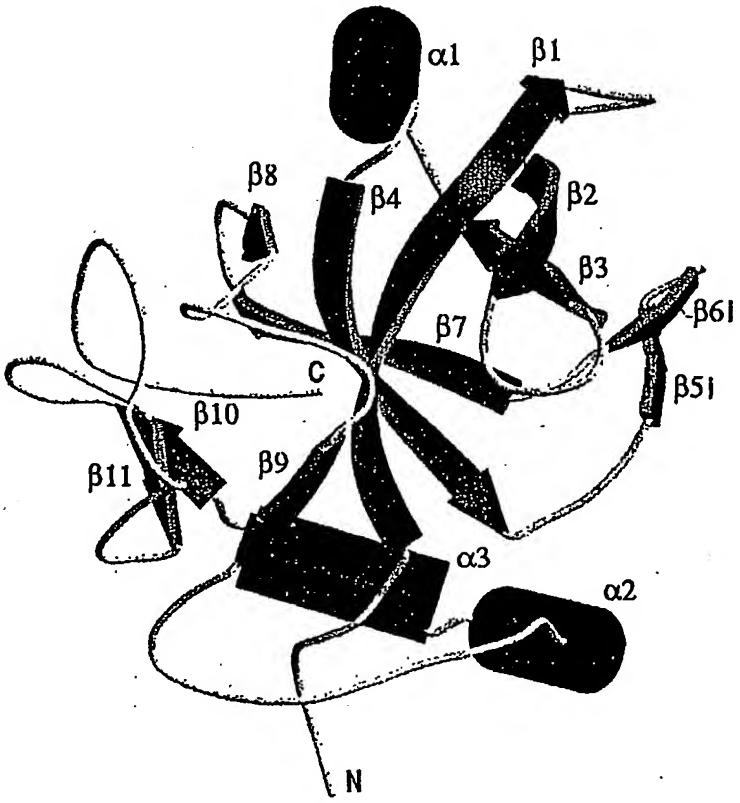

FIG 7

Homologous Structures of EMAPII and Other Cytokines

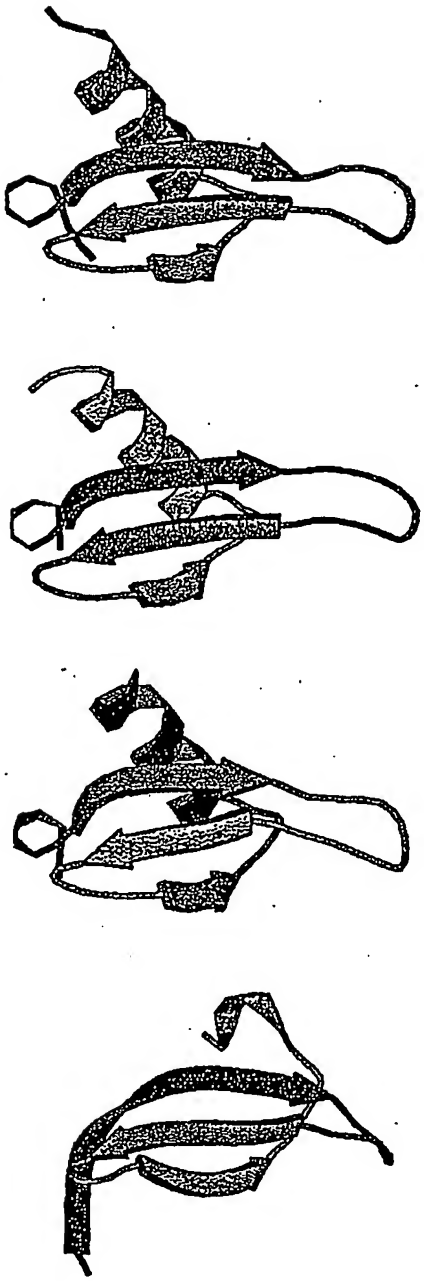

FIG 8

SEQUENCE LISTING

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35 40 45

Arg Val Glu Asn Ala Lys Leu Lys Lys Glu Ile Glu Glu Leu Lys Gln
50 55 60

Glu Leu Ile Gln Ala Glu Ile Gln Asn Gly Val Lys Gln Ile Ala Phe
65 70 75 80

Pro Ser Gly Thr Pro Leu His Ala Asn Ser Met Val Ser Glu Asn Val
85 90 95

Ile Gln Ser Thr Ala Val Thr Thr Val Ser Ser Gly Thr Lys Glu Gln
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Ile Lys Gly Gly Thr Gly Asp Glu Lys Lys Ala Lys Glu Lys Ile Glu
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145                 150                 155                 160

Cys Ile Ile Thr Ala Arg Lys His Pro Asp Ala Asp Ser Leu Tyr Val
                165                 170                 175

Glu Glu Val Asp Val Gly Glu Ile Ala Pro Arg Thr Val Val Ser Gly
            180                 185                 190

Leu Val Asn His Val Pro Leu Glu Gln Met Gln Asn Arg Met Val Ile
        195                 200                 205

Leu Leu Cys Asn Leu Lys Pro Ala Lys Met Arg Gly Val Leu Ser Gln
    210                 215                 220

Ala Met Val Met Cys Ala Ser Ser Pro Glu Lys Ile Glu Ile Leu Ala
225                 230                 235                 240

Pro Pro Asn Gly Ser Val Pro Gly Asp Arg Ile Thr Phe Asp Ala Phe
                245                 250                 255

Pro Gly Glu Pro Asp Lys Glu Leu Asn Pro Lys Lys Lys Ile Trp Glu
            260                 265                 270

Gln Ile Gln Pro Asp Leu His Thr Asn Asp Glu Cys Val Ala Thr Tyr
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INTERNATIONAL SEARCH REPORT

Internati... ...pplication No.
PCT/KR 00/00630

CLASSIFICATION OF SUBJECT MATTER

IPC[7]: A61K 38/17, C12N 15/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC[7]: A61K 38/17, C12N 15/12

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WPI, CAS, STN-registry

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5641867 A (STERN et al.) 24 June 1997 (24.06.97) figures 4A-4D; column 2, lines 18-49; column 4, lines16-21; column 12, lines 44-65; column 30, line 31- column 31, line 67.	1,2,5
A	WO 00/29620 A1 (CHILDREN´S HOSPITAL OF LOS ANGELES) 25 May 2000 (25.05.00) page 2; claims 7,13,17.	5-8
A	PARK, SG., Precursor of pro-apoptotic cytokine modulates aminoacylation activity of tRNA synthetase, J Biol Chem 1999 Jun 11; 274(24):16673-6 (abstract) Medline, [retrived on 30.01.2001]. Retrieved from the Internet: < URL: http://130.14.32.42/cgi-bin/VERSION_B/IGM-client?6655+records+1	1,5

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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Date of the actual completion of the international search	Date of mailing of the international search report
31 January 2001 (31.01.2001)	30 March 2001 (30.03.2001)
Name and mailing adress of the ISA/AT Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Authorized officer MOSSER Telephone No. 1/53424/437

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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 00/00630

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	SCHWARZ et al. Endothelial-monocyte activating polypeptide II, a novel antitumor cytokine that suppresses primary and metastatic tumor growth and induces apoptosis in growing endothelial cells. J. Exp. Med. 1999, 190(3), 341-353 (Eng.). Columbus, OH, USA: Chemical abstracts, Vol. 131, No. 19, 8 November 1999, page 554, the abstract No. 256154f.	1,5,6

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 00/00630

Patent document cited in search report			Publication date	Patent family member(s)			Publication date
SC	A	HWARZETA L.ENDOTH		none			
US	A	5641867	24-06-1997	AU	A1	79615/94	18-04-1995
				CA	AA	2172729	06-04-1995
				EP	A1	721463	17-07-1996
				EP	A4	721463	25-06-1997
				JP	T2	9505987	17-06-1997
				WO	A1	9509180	06-04-1995
WO	A	0029620		none			